Effective Enrichment of Murine Epidermal Langerhans Cells by a Modified (Mismatched) Panning Technique

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A method for the enrichment of murine epidermal Langerhans cells (LC) is described in detail. It is based on positive selection of LC from pre-enriched fresh or cultured epidermal cell suspensions derived from ear skin by a modified panning technique. The method uses the interspecies cross-reactivities of anti-immunoglobulin antibodies: when LC in an epidermal cell suspension are labeled with mouse anti-major histocompatibility complex (MHC) class II antibodies they bind to petri dishes coated with anti-rat immunoglobulin antibodies. We therefore call this method "mismatched panning." After rinsing off non-adherent cells, the adherent LC can easily be dislodged by adding excess amounts of rat immunoglobulins, which effectively compete with the LC-bound mouse anti-MHC class II antibodies for binding to the petri dish.

Using this modified panning technique, both fresh and cultured LC could be enriched up to more than 90% purity. From one ear, $2.0-3.0 \times 10^4$ fresh LC and $3.0-4.5 \times 10^4$ cultured LC could be obtained. Of all LC present in a primary, unenriched epidermal cell suspension, 40–60% were recovered when panned immediately after isolation of the epidermal cells and 50–75% when panned after 3 d of epidermal cell culture. Viability of panned LC was consistently more than 90%. Antigen presenting and T-cell–stimulating capacity of LC and responses to the cytokines granulocyte/macrophage colony-stimulating factor and tumor necrosis factor-α were not impaired by this panning procedure.

The major advantage of this method compared to pre-existing panning techniques is the ease with which adherent LC can be dislodged from the panning dishes. Because the elution procedure is very gentle, virtually all panned LC are viable. As a consequence, good yields of highly enriched LC can be obtained in a reasonable time. *J Invest Dermatol 99:803–807, 1992*

Freshly isolated murine Langerhans cells (fLC) are weak stimulators of resting T cells, but they develop into powerful immunostimulatory dendritic cells (DC) during 2–3 d of epidermal cell (EC) culture [1]. Fresh LC are, therefore, considered to be immature elements of the DC system [2]. They are a promising model for unraveling the unique accessory signals needed for the stimulation of resting T cells, i.e., the initiation of primary T-cell–dependent immune responses [2]. Such studies often need to be done with highly purified LC populations. They are hampered by the fact that the purification of LC, which constitute only 1–3% of all EC, is difficult and an LC line is not currently available. Several different approaches have been taken to enrich LC (reviewed in [3]). Negative selection techniques do not allow reproducible enrichment LC of the required purity [3–9]. Positive selection by fluorescence-activated cell sorting (FACS) is time consuming; it depends on expensive equipment and the viability of sorted cells may suffer [10–17]. Alternatively, positive selection by panning, is tricky and problems may occur when eluting the adherent LC from the panning dishes [12,18–24]. To avoid these problems we developed an improved panning technique that allows the effective enrichment of viable LC from both freshly prepared and cultured EC suspensions.

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Abbreviations:
cLC: cultured LC
DC: dendritic cell(s)
EC: epidermal cell(s)
FACS: fluorescence activated cell sorter
FCS: fetal calf serum
FITC: fluorescein isothiocyanate
fLC: freshly isolated LC
GM-CSF: granulocyte/macrophage colony-stimulating factor
Ig: immunoglobulin(s)
LC: epidermal langerhans cell(s)
MoAb: monoclonal antibody
PBS: phosphate-buffered saline without calcium and magnesium
TNF-α: tumor necrosis factor-α

MATERIALS AND METHODS

Mice BALB/c and C3H/He/N mice were purchased from Charles River Wiga (Sulzfeld, Germany) and used at 6–10 weeks of age.

Media and Reagents The culture medium used throughout was RPMI-1640 (Seromed-Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS) (Gibco Laboratories, Paisley, Scotland), 50 μM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), 200 mM L-glutamine (Gibco), and 20 μg/ml gentamicin (Gibco). Recombinant murine granulocyte/macrophage colony-stimulating factor (GM-CSF; specific activity 4×10^7 U/mg) was kindly provided by Dr. S. Gillis, Immunex Corporation, Seattle, WA; recombinant murine tumor necrosis factor-α (TNF-α; specific activity 2.6×10^7 U/mg) was a gift of Dr. G. R. Adolf, Bender Co., Vienna, Austria.

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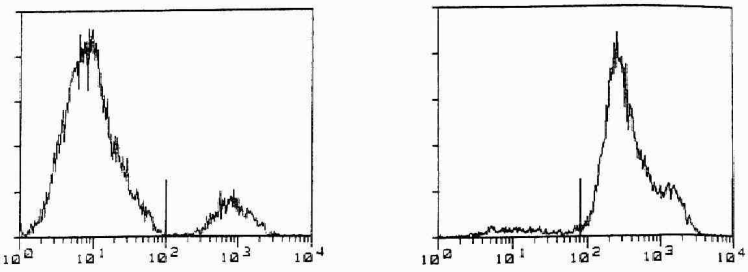

Figure 1. Monitoring of LC enrichment in a typical experiment by flow cytometry. The primary EC suspension from 100 ears yielded 200 × 10^6 EC (~90% viable) containing 3% LC. After pre-enrichment 20 × 10^6 EC (~95% viable) remained showing 15% LC *(left)*. "Mismatched panning" resulted in a cell population (2 × 10^6, ~95% viable) consisting of 92% LC *(right)*. LC were panned with MoAb HB32 (anti-I-$E^{k,d}$) and stained with biotinylated MoAb B21-2 (anti-I-$A^{b,d}$) followed by streptavidin FITC. *Vertical bars*, background staining with isotype-matched control antibody.

Preparation of EC and Pre-Enrichment of LC EC suspensions (viability >85%) containing 1–3% LC were prepared from ear epidermis by standard trypsinization [3,9,25]. Pre-enrichment of fLC was achieved by removing the majority of both keratinocytes and dendritic epidermal T cells [26,27] by treatment with anti-Thy-1.2 monoclonal antibody (MoAb) and complement exactly as described [3,16]. For pre-enrichment of cultured LC (cLC), the non-adherent fraction of EC cultured for 72 h was floated on dense bovine serum albumin columns exactly as described previously [3,25].

Final Enrichment by "Mismatched Panning" After pre-enrichment, EC were treated for 30 min at 4°C with culture supernatants of one of the following MoAbs: anti-I-$E^{k,d}$ (mouse IgG2a, clone 14-4-4S, HB32, ATCC), anti-I-$A^{b,d}$ (rat IgG2b, clone B21-2, TIB229, ATCC), or anti-CD45 (rat IgG2b, clone M9/1, TIB122, ATCC). Final concentration of EC was 2 × 10^6/ml; supernatants were diluted 1:5 with culture medium.

After two washes with culture medium, cells were transferred onto bacteriologic grade petri dishes that, in contrast to published panning techniques [18–24], had been coated with goat anti-immunoglobulin (Ig) antibodies that were *mismatched*, i.e., for panning of LC treated with mouse MoAb, goat anti-rat Ig was used; for panning of LC treated with rat MoAb, goat anti-mouse Ig was used. To coat petri dishes (100 mm, number 1029, Falcon), 40 μg of goat anti-Ig (AffiniPure goat anti-rat IgG [H + L], code 112-005-003, Jackson Immunoresearch Laboratories, West Grove, PA or goat anti-mouse IgG + IgM [H + L], code 4153, TAGO, Burlingame, CA) were diluted in 10 ml PBS and dishes were incubated with this solution for 1 h at room temperature, followed by extensive rinsing with several changes of PBS. Antibody-coated dishes were normally prepared before use; they could, however, be stored in PBS overnight. Storage conditions were not tested any further.

For panning 10 to 30 × 10^6 cells were loaded onto one anti-Ig-coated dish in 10 ml culture medium and incubated for 30 min at room temperature. Adherence of cells to the dishes was checked by phase contrast. If necessary, dishes were placed at 37°C for a further 10 min. Non-adherent cells were gently rinsed off the dish with pre-warmed PBS and complete removal was assessed under the inverted microscope. Finally, adherent cells (*i.e.*, LC) were released by adding excess amounts of gamma globulin matched to the antibody bound to the petri dish: in the case of anti-rat Ig-coated dishes, we used rat gamma globulin (Jackson, code 012-000-002); in the case of anti-mouse Ig-coated dishes, we applied mouse gamma globulin (Jackson code 015-000-002). Dishes were incubated with either reagent at a concentration of 0.5 mg/ml culture medium; 10 ml of ice-cold gamma globulins were added to each dish and incubated for 10 min at room temperature. Due to the higher affinity of the plate-bound anti-Ig to the matched gamma globulins, there was an effective competition for the binding of LC to the dish. As a consequence, they could easily be dislodged by gentle pipetting, thus providing a highly viable and highly enriched LC population. To reduce extensive cell losses, the detached LC were collected in 15 ml polypropylen tubes (number 2097, Falcon) that had been pre-incubated with culture medium. The first centrifugation was done with 460 × *g*.

Assessment of LC Purity, Viability, and Function LC were recognized by staining with biotin or fluorescein isothiocyanate-conjugated (FITC) anti-MHC class II MoAbs (HB32-FITC and B21-2-FITC) and analyzed by flow cytometry using a FACScan instrument (Becton-Dickinson, Mountain View, CA). Dead cells were labeled by propidium iodide and gated out. In addition, cLC could also be identified by their characteristic hairy and "veiled" morphology in the hemocytometer, in which viability was determined by trypan blue exclusion. Oxidative mitogenesis assays [16,25] and antigen-specific hybridoma assays [28] were done as described.

RESULTS

"Mismatched Panning" of Fresh LC Freshly prepared EC suspensions yielded approximately 2 × 10^6 EC per ear. This number corresponds well with previously published values [25]. Viability was consistently above 85%. Primary EC suspensions contained 1–3% LC as determined by anti-MHC class II immunolabeling.

Fresh EC suspensions pre-enriched for LC by removal of keratino-

Table I. Purification of Murine Epidermal Langerhans Cells—Compared with Published Procedures[a]

			Fresh LC[d]						
	Cultured LC	Fresh LC	Koch et al [16]	Witmer Pack et al [18]	Koyama et al [20]	Inaba et al [13]	Sullivan et al [15]	Picut et al [17]	Becker et al [22]
LC Enrichment	95.4 ± 2.7%	91.3 ± 2.2%			95%			98%	
Range	85–98%	87–95%	76–92%	80–85%		95–99%	94–99%		72–86%
LC Viability	>95%	>85%	>90%	NI	90%	NI[c]	94–99%	80%	>95%
LC (number/ear)	3.6 ± 0.4	2.6 ± 0.3	NI	NI	BS	NI	BS	BS	BS
Range	3.0–4.5	2.0–3.0	0.5–1.0	0.5–1.5	BS	NI	BS	BS	BS
LC yield[b]	50–75%	40–60%	20–30%	NI	NI	NI	39%	71%	NI

[a] Fresh BALB/c LC were panned as described in *Materials and Methods* (number of experiments: 8 for Cultured LC, 14 for fresh LC).
[b] LC yields, LC recovered after the enrichment procedure as a percentage of LC present in the original primary epidermal cell suspension.
[c] NI, not indicated; BS, body wall skin.
[d] Comparative data from the literature involve panning [16,18,20], cell sorting [13,15,17], and magnetic bead [22] approaches.

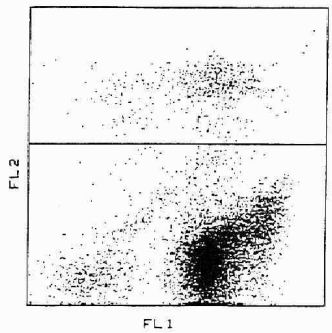

Figure 2. Determination of LC viability by flow cytometry. LC were panned with MoAb HB32 (anti-I-$E^{k,d}$). The eluted LC-enriched fraction was stained with FITC-anti-I-$A^{b,d}$ to identify LC (*horizontal axis,* FL1) and propidium iodide to label dead cells (*vertical axis,* FL2). Data are presented as a dot plot. FACS statistics showed that 93% of all cells were I-A-positive, *i.e.,* were LC, of which 88% did not stain with propidium iodide, indicating that they were viable.

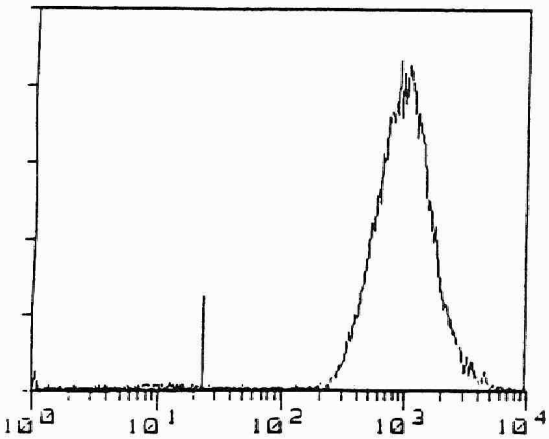

Figure 4. FACS analysis of cultured panned LC. LC were panned with MoAb B21-2 (anti-I-$A^{b,d}$). The eluted LC-enriched fraction was stained with FITC anti-I-$E^{k,d}$ (HB32) to identify LC. FITC fluorescence on *horizontal axis;* number of events on *vertical axis. Vertical bar,* background staining with isotype-matched control antibody. FACS statistics indicated 97.7% I-E-positive cells, *i.e.,* LC.

cytes and dendritic epidermal T cells by anti-Thy-1 plus complement and subsequent trypsin/DNAse treatment [3,16] were highly viable (>90%). The percentage of LC ranged between 10% and 28% (Fig 1).

After this mismatched panning, fresh LC were consistently enriched to about 90% (Table I). The degree of enrichment was assessed by FACS analysis (Figs 1 and 2) and in some experiments by immunofluorescence staining of cell suspensions (see Fig 3). Most LC were viable as determined by trypan blue exclusion and propidium iodide analysis (Fig 2).

Assuming 2-3% LC in a freshly prepared EC suspension, at least 40% and up to 60% of the initial LC were recovered in the final, highly enriched cell population using the mismatched panning approach. Fresh LC were routinely panned with MoAb HB32 (anti-I-$E^{k,d}$). Similar results (not shown) were obtained with MoAb TIB122 (anti-CD45). In our study, MoAb B21-2 (anti-I-$A^{b,d}$) produced lower yields and was therefore not used for panning of fresh LC.

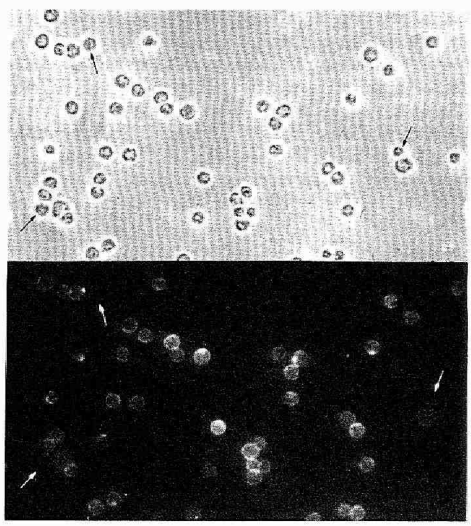

Figure 3. Immunofluorescence analysis of freshly isolated panned LC. LC were panned with MoAb HB32 (anti-I-$E^{k,d}$), eluted from the dish, and stained in suspension with MoAb B21-2 (anti-I-$A^{b,d}$), followed in sequence by biotinylated anti-rat Ig and streptavidin FITC. Note that most of the cells apparent in phase contrast *(top)* are I-A-positive *(bottom), i.e.,* LC. Contaminating I-A-negative keratinocytes are marked with arrows, magnification ×100.

"Mismatched Panning" of Cultured LC After 3 d of EC culture, non-adherent cell fractions contained a large percentage of dead cells. Pre-enrichment of these suspensions by passage over dense albumin columns gave floating fractions containing virtually all LC at 40-75% purity [3,25].

After mismatched panning, cultured LC were consistently enriched to about 95% (Table I, Fig 4). In addition to flow cytometry, LC could easily be recognized and counted in the hemocytometer due to their characteristic veiled or hairy shape. It should be noted that although cLC expressed much higher levels of MHC class II molecules than fLC, and therefore adhered to the dishes much tighter, they could equally well be eluted from the anti-Ig-coated dishes on the addition of excess amounts of gamma globulins.

Relating the numbers of cLC obtained by panning to the numbers of LC present in the primary EC suspension, at least 50% and up to 75% of LC could be recovered.

Effects of Cytokines on the Viability of Panned LC The cytokines GM-CSF and TNF-α sustain the viability of LC in short-term culture [6,16,18]. Highly purified fresh LC obtained by mismatched panning rapidly died in culture in the absence of exogenously added cytokines. The addition of GM-CSF (100-200 U/ml) or TNF-α (125 U/ml) to the cultures kept LC alive and resulted in survival rates of approximately 50% (not shown) as previously described [6,16,18]. This drop in LC viability may be used as a sensitive functional indicator for the purity of panned LC. If LC enrichment is below approximately 75%, enough GM-CSF-producing keratinocytes are present in the cultures to ensure LC survival in the absence of exogenously added cytokines [16]. Using this functional readout in addition to the panning experiments evaluated by flow-cytometric quantification of LC (Table I), we have recorded more than 40 panning experiments with LC enrichment higher than 75%.

Table II. Effects of Panning on the Antigen-Presenting Capacity of Langerhans Cells[a]

Type of LC Enrichment	Antigen Added to Assay	Proliferation of T Cells in cpm × 10^{-3} at LC Doses of				
		3×10^3	10^3	3×10^2	10^2	None
Hybridoma Assay for Freshly Isolated LC						
A. Pre-enriched	None	0.6	0.7	0.6	0.7	0.4
B. Pre-enriched	myoglobin	118.2	49.2	23.8	9.6	0.8
C. HB32 panned	None	0.5	0.4	0.4	0.4	
D. HB32 panned	myoglobin	121.3	72.1	18.5	8.2	
Oxidative Mitogenesis Assay for cLC						
E. Pre-enriched	None	149.8	84.2	30.2	8.9	
F. B21-2 panned	None	162.1	66.4	23.1	10.2	
G. HB32 panned	None	148.6	76.3	29.8	9.7	

[a] Both freshly isolated LC and cultured LC were either only pre-enriched, or panned as described in *Materials and Methods*. Note that panning does not impair the processing and presenting function of LC (*B* versus *D, underlined*). Graded doses of cLC were co-cultured with 3×10^5 syngeneic periodate-modified T cells for 24 h in an oxidative mitogenesis assay. Both populations of panned cLC *(rows F and G)* as well as cLC pre-enriched by flotation *(row E)* elicit comparable T-cell proliferation.

Unimpaired Functional Capacities of Panned LC MHC class II molecules are essential elements of the immune response. We therefore checked whether panning via these surface molecules would impair the accessory cell functions of LC. Because freshly isolated LC are poor stimulators of resting T cells, we chose to monitor the antigen-presenting capacity of panned LC to peptide-specific T-cell hybridomas rather than to resting T cells. Panned LC processed and presented sperm whale myoglobin to the peptide-specific hybridoma as effectively as equal numbers of pre-enriched fresh LC that had not been exposed to the anti-MHC class II MoAb used for panning (Table II). The effects of panning on cultured LC were assessed in an oxidative mitogenesis assay [16,25]. The panning procedure did not diminish the T-cell–stimulatory capacity of panned cLC as compared to cLC pre-enriched by floatation on dense albumin columns (Table II).

DISCUSSION

Enrichment of trace cell populations can be achieved by negative or positive selection techniques (reviewed in [3]). It is difficult to obtain LC at a reasonable degree of purity and in sufficient numbers by negative selection. Positive selection, such as panning [12,16,18–24] and fluorescence-activated cell sorting (FACS) [11–17], has been successfully applied for the enrichment of LC.

Problems Associated with Positive Selection Methods FACS purifications are generally time consuming and as a consequence cell viability may drop. Therefore, panning approaches are often preferred. The principle of panning [29] is to label the desired cells with a specific antibody generated in mouse, rat, rabbit, *etc.* and to make the labeled cells bind to dishes coated with the respective anti-mouse, anti-rat, anti-rabbit Igs. In this "species-matched" combination of antibodies the main problem is to elute the bound cells from the dishes. This is achieved either mechanically by means of a rubber policeman [22] or by adding an excess amount of species-matched immunoglobulin [18]. In the former case, the viability of the detached cells may suffer. In the latter, the following dilemma may occur: if the cells stick too firmly they will not come off well; if they stick too loosely, they may be washed away with the non-adherent cells and the yields will be low.

Principle of the "Mismatched Panning" Method We tried to circumvent the problem of cells adhering either too firmly or too loosely by modifying the panning technique of Witmer-Pack et al [18] and using "species-mismatched" combinations of antibodies. The idea was based on the fact that conventionally prepared antibodies against immunoglobulins of a certain species normally cross-react well with immunoglobulins of a closely related species. For example, an anti-mouse IgG antibody binds well to rat IgG, and vice versa. Therefore, we panned LC with a species-mismatched combination of antibodies: LC were tagged with a mouse monoclonal antibody and bound to dishes coated with anti-rat antibodies. LC adhered tightly to the dishes. Elution of LC was easily and efficiently accomplished by adding excess amounts of rat immunoglobulin. Because the dish-bound anti-rat Ig has a higher affinity to the competing rat immunoglobulins than to the LC-bound mouse monoclonal antibody, the LC detached instantly.

Technical Points It should be noted that some factors may be crucial to the final outcome and should be considered in case of unsatisfactory results. First, we found that some batches of trypsin may be too aggressive and deteriorate binding of LC to the dishes. Second, care has to be taken to choose an anti-Ig antibody that cross-reacts sufficiently well with the other, "mismatched" species. There may be variability from batch to batch or between different manufacturers. Third, the bacteriologic petri dishes are frequently defective in their surface properties. Sharply circumscribed areas on one dish or whole dishes cannot be coated with antibody and, as a consequence, no cells will adhere.

Evaluation of the Mismatched Panning Method Enrichment of cells by panning avoids the need for expensive technical equipment and it is less time consuming than sorting. The specific advantages of the mismatched panning method are 1) elution of adhering cells from the dishes is no problem, and therefore the adherence of LC to the dish may be maximized by incubation at room temperature or by prolongation of incubations if needed. Even cLC that adhere to the dishes very tightly due to their high expression of MHC class II molecules can be easily detached. 2) Because of the gentle elution procedure and the relatively short duration of the procedure compared to cell sorting [13,15,17], virtually all panned LC remain viable. 3) As a consequence, the yields of highly enriched, viable LC per mouse are about twice those described for matched pans ([18,22]; Table I). Finally, the method is versatile in that it may be applied to different cell types employing different combinations of antibodies.

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